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Carnosine and Related Compounds Protect Against Copper-Induced Damage of Biomolecules

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At concentrations of 1 mM, the protective effects of carnosine and related compounds including anserine, homocarnosine, histidine, and β-alanine were investigated against copper-catalyzed oxidative damage to deoxyribose, ascorbic acid, human serum albumin, liposome, and erythrocytes. Carnosine and anserine reduced Cu (II) to bathocuproine-reactive Cu (I) in a time- and a dose-dependent manner while the others did not. Carnosine reduced 86% of 100 μM Cu (II) in 60 min. Carnosine, homocarnosine, anserine, and histidine inhibited copper-catalyzed deoxyribose degradation by 75, 66, 65, and 45%, respectively. In the presence of 1 μM Cu (II), carnosine and related compounds inhibited ascorbic acid oxidation by 55–85% after incubation for 20 min. In the presence of 0.15 mM ascorbic acid and 0.8 mM H_2O_2, carnosine, anserine, homocarnosine, and histidine inhibited copper-catalyzed oxidation of human serum albumin by 41, 21, 29, and 24%, respectively, as determined by carbonyl formation. These compounds also significantly inhibited copper-catalyzed liposomal lipid peroxidation as measured by malondialdehyde and lipid hydroperoxides. Carnosine, anserine, homocarnosine, and histidine inhibited hemolysis of bovine erythrocytes induced by 0.1 mM Cu (II). These results suggest that histidine-containing dipeptides may play an important role in protecting against free radical-mediated tissue damage.

Keywords: Antioxidant, Ascorbic acid, Carnosine and related compounds, Hemolysis, Lipid peroxidation.

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Introduction

Histidine-containing dipeptides such as carnosine, anserine, and homocarnosine are present in considerable amounts in several vertebrates tissues including skeletal muscle, eye, olfactory system, and brain (Crush, 1970; Flancbaum *et al.*, 1990; Jackson and Lenney, 1996). Several physiological functions of these dipeptides have been postulated. Carnosine has a buffering effect by neutralizing lactic acid produced in skeletal muscle (Harris *et al.*, 1990). Carnosine and anserine are effective copper-chelating agents and may play a role in copper metabolism *in vivo* (Brown, 1981). These dipeptides function as neurotransmitters in the olfactory bulbs, as physiological activators for myosine ATPase, and as regulators of other enzymes (Parker and Ring, 1970; Ikeda *et al.*, 1980). At physiological concentrations, these dipeptides also have anticarcinogenic, antiglycating, and antiaging effects by acting as antioxidants (Hipkiss *et al.*, 1998).

Oxygen radicals have been implicated as an important cause of oxidative modification of biological molecules such as proteins, lipids, carbohydrates, and nucleotides (Koh *et al.*, 1997; Lee and Hendricks, 1997a; 1997b; Kim *et al.*, 1998; Lee *et al.*, 1998a; 1998b). The oxidative degradation of these molecules can be catalyzed by transition metal ions (Lee and Hendricks, 1997a; Kim *et al.*, 1998). Carbonyl formation as an early marker for protein oxidation and lipid peroxidation products is increased in free radical-related diseases such as rheumatoid arthritis, ischemia-perfusion injury, and atherosclerosis (Davies, 1987; Chapman *et al.*, 1989; Cochrane, 1991; Halliwell, 1991; Reznick *et al.*, 1992). Antioxidants, vitamin C and E, and metal-chelating agents such as desferrioxamine protect against these free radical-mediated tissue injuries (Cochrane, 1991; Halliwell, 1991; Reznick *et al.*, 1992; Park and Song, 1994).

Erythrocytes and/or their membranes have been used as a model system to study oxidative damage (Shinar *et al.*, 1989). The production of free radicals is also associated with red blood cell-related diseases such as thalassemia, sickle cell anemia, and glucose-6-phosphate dehydrogenase (G6PDH) deficiency (Hebbel, 1985; Rachmilewitz *et al.*, 1985). Free radicals inflict cellular damage leading to the destruction of red blood cells (RBC). In sickle cell anemia, RBC lesions result from extensive oxidative damage induced by excess iron deposition in the membrane (Kuross and Hebbel, 1988). A wide variety of oxidizing agents have been used to induce changes in normal RBC structure and function. Incubation of RBC with trace metals such as iron and copper causes hemolysis by interfering with the generation of NADPH mediated by G6PDH in normal as well as in G6PDH-deficient cells (Shinar *et al.*, 1989).

Although the antioxidant activity of carnosine and related compounds has been illustrated in several model systems, little is known about their comparitive antioxidant activity in the same model systems. In this study, although the content of carnosine and related compounds varies in tissues or organs of mammals, 1 mM concentrations of test compounds were used because the concentration is representative for most of the tissues or organs. We examined the reducing activity of carnosine and its related compounds on Cu (II) in aqueous solutions. We also investigated the protective effects of these compounds against copper-induced oxidative damage to biomolecules including deoxyribose, ascorbic acid (AA), human serum albumin (HSA), and phospholipid as well as erythrocytes as a whole cellular system.

Materials and Methods

Materials L-carnosine, homocarnosine, L-anserine, L-histidine, β-alanine, glutathione, 2-deoxyribose, 2-thiobarbituric acid (TBA), trichloroacetic acid (TCA), tetraethoxypropane (TEP), ascorbic acid, human serum albumin, and phosphatidylcholine (PC) were purchased from Sigma Chemical Co. (St. Louis, USA). Heparinized bovine blood was obtained from the animal physiological laboratory at Utah State University. All solutions were prepared in chelax-treated phosphate buffer (pH 7.4) using water passed through a four-stage Milli Q system equipped with a 0.2 μm pore-sized final filter.

Cuprous ion measurement The reduction of Cu (II) was assessed by following the appearance of bathocuproine-reactive Cu (I) (Williams *et al.*, 1977). The reaction mixtures in 0.1 M sodium phosphate buffered saline (PBS, pH 7.4) included 25, 50, or 100 μM of Cu (II), test compound solutions, and 0.2 mM bathocuproine sulfonic acid. The reaction was initiated by adding Cu (II) to the reaction mixtures and the absorbance was monitored spectrophotometrically at 485 nm for 60 min at room temperature. The concentration of Cu (I) was calculated using a 100 μM Cu (I) standard reduced by the addition of 1 mM AA as a reductant for Cu (II).

Deoxyribose damage (hydroxyl radical scavenging) Deoxyribose degradation caused by hydroxyl radicals was determined by the formation of thiobarbituric acid reactive substances (TBARS) (Lee and Hendricks, 1997a). The reaction mixtures (1.0 ml) contained 0.1 M phosphate buffer (pH 7.4), 7 mM deoxyribose, 50 μM Cu (II), 100 μM AA, and test solutions. The mixtures were incubated for 60 min at 37°C. One milliliter of a stock solution [1% (w/v) TBA in 50 mM NaOH plus 2.8% (w/v) TCA] was added to the reaction mixture. The mixture was then heated for 10 min in a boiling water-bath, cooled with tap water, and the absorbance of the pink chromogen was read spectrophotometrically at 532 nm. TBARS concentrations were calculated from a standard curve of malondialdehyde (MDA), a breakdown product of TEP.

Ascorbic acid measurement The reaction mixtures included 0.1 M potassium phosphate buffer (pH 7.4), 100 μM AA, 1 μM Cu (II), and 1 mM test solutions. After the final addition of AA to the reaction mixture, AA was monitored directly by reading the absorbance at 265 nm, and the amount of oxidized AA was calculated from the initial value for 100 μM AA.

Lipid peroxidation Liposomes were prepared by diluting 500 mg PC in 5 ml of chloroform. The chloroform solution was dried under vacuum and resuspended in 100 ml of degassed, argon-saturated 0.1 M potassium phosphate buffer (5 mg PC/ml). The suspension was sonicated for 10 pulses of 30 s under argon. The PC liposome preparations were stored at 4°C under an argon atmosphere until used (Lee and Hendricks, 1997a). The pH of the PC liposome was adjusted to 7.4 immediately prior to use. The reaction mixtures (1.0 ml) containing 0.1 M potassium phosphate buffer (pH 7.4), 2.5 mg PC, 10 μM Cu (II), 100 μM AA, and test solutions were incubated for 90 min at 37°C, and 0.8 ml of the incubated mixtures was used for the TBARS determination (Lee and Hendricks, 1997a). Two milliliters of a 15% TCA-0.375% TBA-0.025 N HCl stock solution was added to 0.8 ml of the incubated liposome. The mixtures were then heated for 10 min in a boiling water bath (95–100°C) to develop the pink color, cooled with tap water, and centrifuged for 20 min at 3500 × *g*. The supernatant containing the pink chromogen was quantitated at 532 nm using a UV 2100 U spectrophotometer (Shimadzu Co., Kyoto, Japan). MDA was calculated from a standard curve of MDA, a breakdown product of TEP.

To remove H_2O_2, 0.1 ml of the incubation mixtures described above was mixed with 50 μl (140 IU) of catalase. After standing for 30 min at room temperature, 0.9 ml of a 90% methanol reagent solution (0.1 mM xylenol orange, 0.25 mM ferrous ammonium sulfate, 0.4 mM butylated hydroxytoluene, 25 mM H_2SO_4) was added to the solution. After 30 min standing at room temperature, the absorbance at 560 nm was measured, and lipid peroxides were calculated by a molar absorption coefficient, $4.5 \times 10^4\ M^{-1}cm^{-1}$ (Wolff, 1994).

Protein damage The reaction mixture (1.0 ml) in 0.1 M phosphate buffer (pH 7.4) containing 2 mg HSA/ml, 50 μM Cu (II), 150 μM AA, 0.8 mM H_2O_2, and 10 mM test solutions were incubated for 60 min at 37°C. After 1 ml of 20% TCA (w/v) was added to the reaction mixture, the solution was centrifuged for 10 min at 1000 × *g*, and the supernatant was discarded. After adding 1 ml of 10 mM 2,4-dinitrophenylhydrazine (DNPH) in 2.5 M HCl, the pellet was gently broken with a glass rod and allowed

to stand for 60 min at room temperature, with vortexing every 10–15 min. Then, 1 ml of 20% TCA was added and centrifuged for 10 min at 1000 × *g*. The pellet was obtained and washed with 2 ml of 10% TCA twice and then with 1 ml of ethanol-ethyl acetate (1:1) three times to remove the free DNPH. The protein precipitates were dissolved in 1 ml of 6 M guanidine hydrochloride solution and left for 10 min at 37°C with general vortex mixing. Absorbance at 370 nm was read against a blank of the guanidine hydrochloride solution, and carbonyl content was calculated using the molar absorption coefficient of 22,000 M^{-1} cm^{-1} (Reznick and Parker, 1994).

Hemolysis, Hemoglobin, and Methemoglobin (MetHb)/ Oxyhemoglobin (OxyHb) Bovine blood was centrifuged for 15 min at 1000 × *g*, and the supernatant including the buffy coat was discarded by aspiration. Erythrocytes were washed three times with 5 vol of 10 mM PBS (pH 7.4). The reaction mixtures containing 10% (v/v) bovine erythrocyte suspension in 10 mM PBS (pH 7.4), 100 μM Cu (II), 1 mM AA, and 1 mM test solutions were incubated for 2 or 6 h at 37°C. For hemolysis analysis, 0.1 ml of the incubation mixtures (6 h incubation) was taken, hemolyzed with 5 ml of deionized water, vortexed vigorously, and measured spectrophotometrically at 575 nm as 100% hemolyzed control. After the remaining 0.9 ml of the incubation mixture was centrifuged at 1000 × *g* for 10 min at 5°C, 0.1 ml of the supernatant was diluted with 5 ml of deionized water and the absorbance was measured at 575 nm (Brownlee *et al.*, 1977). For the measurement of hemoglobin release and the ratio of MetHb/OxyHb, the reaction mixture was centrifuged at 1000 × *g* for 10 min. Twenty microliters of the supernatant was used for the determination of the level of hemoglobin using the Sigma commercial kit for plasma hemoglobin. The precipitates were hemolyzed with 8 ml of deionized water, and the absorbances were measured at 540 nm for OxyHb and 630 nm for MetHb (Shinar *et al.*, 1989).

Results

Reduction of Cu (II) Bathocuproine forms a complex with Cu (I) which has maximum absorbance at 485 nm. Carnosine and anserine reduced Cu (II) to bathocuproine-reactive Cu (I) in 0.1 M PBS (pH 7.4) (Fig.1). The reducing rate of Cu (II) by 1 mM carnosine or 1 mM anserine was time-dependent. When the standard of 100 μM Cu (I) reduced by excess ascorbic acid was used, 1 mM of carnosine and anserine reduced 86% and 80% of 100 μM Cu(II), respectively, within 60 min at room temperature (Fig. 1). Homocarnosine, histidine, and β-alanine did not affect the reduction of Cu (II) to Cu (I). The reduction of Cu (II) by carnosine was dose-dependent in the presence of 25, 50, and 100 μM Cu (II) (Fig. 2). The reducing activity of carnosine in PBS (pH 7.4) was similar to that in other biological buffers including HEPES, MOPS, and PIPES. However, carnosine and related compounds could not reduce Fe (III) to Fe (II) in the buffer solutions (data not shown).

Deoxyribose degradation (Hydroxyl radical scavenging activity) Addition of 50 μM Cu (II) in the presence of

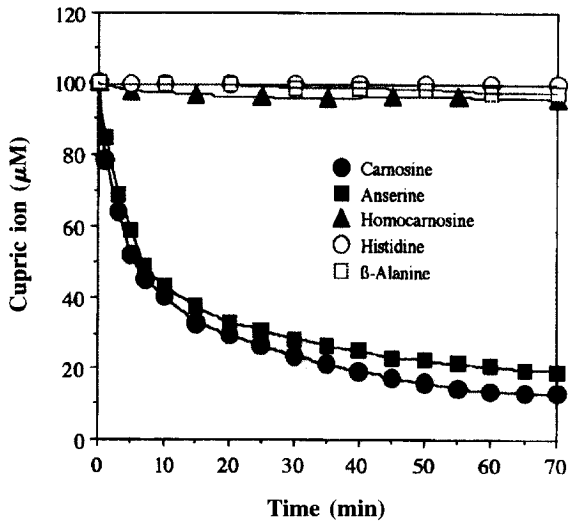

Fig. 1. Reduction of 100 μM Cu (II) to bathocuproine-reactive Cu (I) in 0.1 M PBS (pH 7.4) by 1 mM carnosine and related compounds. The absorbance at 485 nm was monitored for 70 min at room temperature after addition of Cu (II) to reaction mixtures including 1 mM test solution and 0.2 mM bathocuproine sulfonic acid. The Cu (I) concentration was calculated using the absorbance of 100 μM Cu (I) reduced by 1 mM AA. Data points represent the means of three determinations.

100 μM AA to deoxyribose caused degradation of the sugar into an MDA-like compound, which forms a chromogen with TBA (Table 1). Hydroxyl radicals produced via the Fenton reaction are responsible for the breakdown of deoxyribose. Carnosine, anserine, homocarnosine, and histidine effectively inhibited the Cu (II)-catalyzed deoxyribose degradation, indicating a hydroxyl radical scavenging activity of these compounds. β-Alanine did not inhibit the Cu (II)-catalyzed degradation of deoxyribose. At a 1 ml concentration, carnosine, anserine, homocarnosine, and histidine inhibited deoxyribose degradation by 75, 66, 65, and 45%, respectively. The inhibitory effect of these compounds was dose-dependent (data not shown). The histidine-containing dipeptides more effectively inhibited the deoxyribose degradation than did histidine alone. Catalase (50 μg/ml) also effectively inhibited deoxyribose degradation by 62%, probably resulting from removal of H_2O_2 in the solution.

Inhibition of ascorbic acid oxidation The acceleration of AA oxidation by copper is accompanied by the one-electron reduction of molecular oxygen, resulting in the production of oxygen free radicals. When AA concentration was monitored by UV absorbance at 265 nm, 100 μM AA was completely oxidized within 20 min in the presence of 1 μM Cu (II) (Fig. 3). One millimolar carnosine, anserine, homocarnosine, or histidine effectively inhibited the Cu (II)-catalyzed AA oxidation. However, 1 mM β-alanine had no effect on the Cu (II)-catalyzed AA

Table 1. Inhibition by 1 mM carnosine and related compounds of deoxyribose degradation catalyzed by Cu (II) and ascorbic acid (hydroxyl radical scavenging activity).

Reaction mixtures[a]	TBARS (nmol/ml)	% Inhibition
Cu (II) + AA + buffer	4.80 ± 0.25	–
Carnosine	1.21 ± 0.15	74.8
Homocarnosine	1.64 ± 0.16	65.8
Anserine	1.67 ± 0.13	65.2
L-Histidine	2.64 ± 0.14	45.0
β-Alanine	4.87 ± 0.37	0
Catalase (50 μg/ml)	1.83 ± 0.08	61.9

[a] Reaction mixtures containing 7.0 mM deoxyribose in 0.1 M potassium phosphate buffer (pH 7.4), 50 μM Cu (II), 100 μM AA, and 1 mM test solutions were incubated for 60 min at 37°C. TBARS concentrations were determined by the TBA test. Values are the mean ± SD of three determinations.

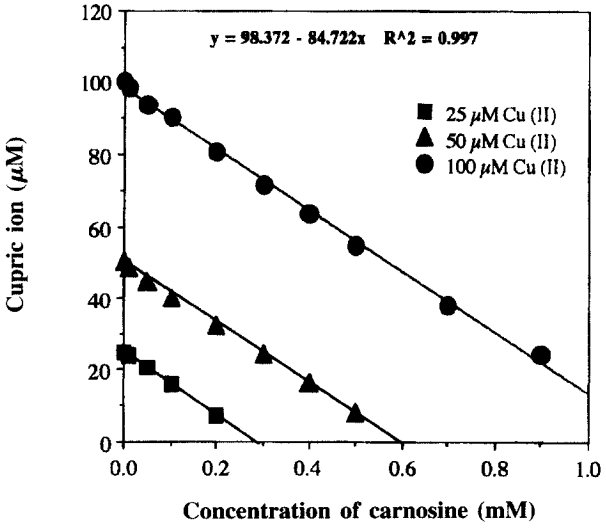

Fig. 2. Effect of various concentrations of carnosine on reduction of Cu (II) to Cu (I) in 0.1 M PBS (pH 7.4) after 60 min standing at room temperature. The absorbance at 485 nm was measured at 60 min after addition of Cu (II) to reaction mixtures including various carnosine concentrations and 0.2 mM bathocuproine sulfonic acid. The Cu (I) concentration was calculated based on the absorbance of 100 μM Cu (I) reduced by 1 mM AA. Data points represent the mean of three determinations.

oxidation. After incubation for 20 min at room temperature, 1 mM carnosine, anserine, homocarnosine, and histidine inhibited AA oxidation by 55, 70, 58, and 85%, respectively. Among these compounds tested, histidine was the most effective for the inhibition of AA oxidation.

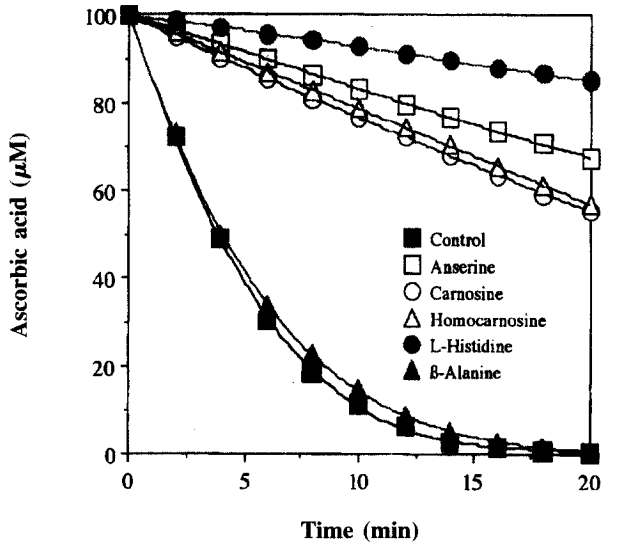

Fig. 3. Inhibition of Cu (II)-catalyzed ascorbate oxidation by 1 mM of carnosine and related compounds. The absorbance of the reaction mixtures in 0.1 M potassium phosphate buffer (pH 7.4), containing 1 μM Cu (II), 100 μM AA, and test solutions was monitored at 265 nm. Data points represent the means of three determinations.

Protection against oxidative protein damage Carbonyl has been used as a marker for the oxidative modification of protein, and metal ions can catalyze protein oxidation. Carbonyl was detected after HSA in 0.1 M phosphate buffer (pH 7.4) was incubated for 60 min at 37°C without reactants. In the presence of 50 μM Cu (II), 150 μM AA and 0.8 mM H_2O_2, carbonyl formation from HSA increased two-fold compared to the control (only HSA) (Table 2). One millimolar carnosine, anserine, homocarnosine, histidine, and glutathione inhibited the Cu (II)-catalyzed oxidation of HSA by 41, 21, 29, 24, and 22%, respectively. Among the tested compounds, carnosine was the most effective inhibitor compound against protein modification. β-Alanine had no effect on the copper-catalyzed protein damage.

Inhibition of liposomal lipid peroxidation In the presence of 10 μM Cu (II) and 100 μM AA, the antioxidant effects of carnosine and related compounds were investigated using PC liposomes (Table 3). Copper catalyzed the liposomal lipid peroxidation, as measured by MDA and lipid peroxides. Without Cu (II) and AA, lipid peroxidation occurred in the PC liposomes, indicating a possible presence of trace amount of metals and preformed lipid peroxides in the reaction mixture. The addition of 10 μM Cu (II) and 100 μM ascorbic acid strongly catalyzed the lipid peroxidation ($p<0.01$). Except for β-alanine, 1 mM carnosine and related compounds strongly inhibited the Cu (II)-catalyzed MDA formation by 64–77 % and the formation of lipid peroxides by 66–88% ($p<0.01$).

Table 2. Protection by 1 mM carnosine and related compounds against Cu (II)-catalyzed oxidative damage to human serum albumin.

Reaction mixtures[a]	Carbonyl (nmol/mg HSA protein)	% vs control
Human serum albumin (HSA)	5.1 ± 0.7	47.2
+ Cu (II) + AA + H_2O_2 + buffer	10.8 ± 1.7	–
+ Carnosine	6.4 ± 0.8	59.3
+ Homocarnosine	7.7 ± 0.9	71.3
+ Anserine	8.5 ± 0.6	78.7
+ L-Histidine	8.2 ± 1.2	75.9
+ β-Alanine	11.0 ± 1.1	101.9
+ Glutathione	8.4 ± 0.4	77.8

[a] Reaction mixtures in 0.1 M phosphate buffer (pH 7.4) containing 2 mg HSA/ml, 50 μM Cu (II), 150 μM AA, 0.8 mM H_2O_2, and test solutions were incubated for 60 min at 37°C and then carbonyl content was spectrophotometrically determined. Data represent the mean ± SD of three determinations.

Table 3. Effect of 1 mM carnosine and related compounds on lipid peroxidation of phosphatidylcholine liposomes.

Reaction mixtures[a]	MDA (nmol/mg PC/ml)	Lipid peroxides (nmol/mg PC/ml)
Liposomes only	0.23 ± 0.07^{b}	3.02 ± 0.36^{b}
+ Cu (II) + AA + buffer	4.61 ± 0.38^{c}	30.93 ± 2.71^{c}
+ Carnosine	1.07 ± 0.16^{d}	4.89 ± 0.64^{d}
+ Homocarnosine	1.64 ± 0.17^{e}	10.67 ± 1.44^{e}
+ Anserine	1.22 ± 0.14^{d}	9.16 ± 1.01^{e}
+ Histidine	1.10 ± 0.10^{d}	3.76 ± 0.57^{bd}
+ β-alanine	4.22 ± 0.41^{c}	31.2 ± 2.28^{c}

[a] Reaction mixtures (final volume, 1.0 ml) in 0.1 M potassium phosphate buffer (pH 7.4) containing 2.5 mg PC, 10 μM Cu (II), 100 μM AA, and test solutions were incubated for 90 min at 37°C. MDA was calculated using a standard curve of TEP. Lipid hydroperoxides contents were calculated by the molar absorption coefficient, 4.5×10^4 $M^{-1}cm^{-1}$, at 370 nm. Data represent the mean ± SD of three determinations.

[bcde] Means within the same column bearing different superscripts are significantly different ($p<0.01$).

Carnosine and histidine were the most effective inhibitors against the formation of MDA and lipid peroxides, respectively.

Protection against hemolysis Oxygen radicals can be produced in solution in the presence of Cu (II) and ascorbic acid. These oxygen radicals can damage erythrocyte membranes, thereby releasing hemoglobin into solution. In the presence of 100 μM Cu (II) and 1 mM AA, bovine erythrocytes released hemoglobin into the reaction solution after incubation for 2 h at 37°C ($p<0.01$). Carnosine and related compounds inhibited membrane breakdown in this preparation (Table 4). Bovine erythrocytes were 30% hemolyzed in the presence of Cu (II) and AA after incubation for 6 h at 37°C. One millimolar carnosine, anserine, homocarnosine, or histidine significantly inhibited the hemolysis ($p<0.01$). The addition of copper and ascorbic acid also induced MetHb formation after incubation for 2 h at 37°C. One millimolar carnosine, anserine, or histidine concentrations significantly increased the MetHb formation compared to the control ($p<0.01$).

Discussion

Carnosine is one of the most abundant nitrous compounds in the non-protein fraction of vertebrate skeletal muscle (ranging 1–20 mM), olfactory epithelium and bulbs (0.3–5.0 mM), and eye lens (Crush, 1970; Boldyrev and Severin, 1990). Carnosine forms a bicyclic ring chain tautomeric structure, resulting in the release of hydrogen ions in solution (Boldyrev *et al.*, 1993). The tautomeric carnosines possess strong biological activity as a quencher of free radicals and as a reducing agent. Carnosine has an anodic cyclic voltammetry response, indicating possible activity as a reducing agent (Kohen *et al.*, 1988). In this study, carnosine and anserine slowly but effectively reduced Cu (II) to Cu (I) in several buffer solutions, whereas homocarnosine, histidine, and β-alanine did not.

Table 4. Effect of 1 mM carnosine and related compounds on Cu (II) + ascorbic acid-induced Hb release, MetHb formation, and hemolysis.

Reaction mixtures[a]	Hemoglobin (mg/dl)	MetHb/OxyHb (%)	Hemolysis (%)
RBC only	2.1 ± 0.7^{b}	3.5^{b}	0.3^{b}
Control (Cu + AA)	29.4 ± 2.7^{c}	33.1^{c}	29.6^{c}
Carnosine	8.1 ± 1.7^{d}	38.4^{cd}	2.5^{d}
Homocarnosine	9.1 ± 1.6^{d}	32.7^{c}	3.5^{d}
Anserine	7.8 ± 1.3^{d}	37.7^{cd}	2.4^{d}
L-Histidine	8.7 ± 1.7^{d}	43.7^{d}	3.3^{d}
β-Alanine	25.2 ± 2.1^{c}	33.5^{c}	26.2^{c}

[a] Reaction mixtures containing 10% bovine erythrocyte suspension in 10 mM PBS (pH 7.4), 100 μM Cu (II), 1 mM AA, and 1 mM test solutions were incubated at 37°C for 2 h. Hemolysis was determined after 6 h incubation against a 100% hemolyzed control. Data represent the mean 6 SD of three determinations.

[bcd] Means within the same column bearing different superscripts are significantly different ($p<0.01$).

The reducing activity of carnosine and anserine was specific for Cu (II) but not for Fe (III), since they could not reduce Fe (III) to Fe (II) in the solutions. The reducing potential of carnosine was about one-tenth that of ascorbic acid. The action of carnosine and anserine may be involved in several enzyme reactions involving copper in biological systems. Further studies will be needed to investigate the role of carnosine in copper-involved enzyme reactions.

Free radicals are involved in the etiology of a number of degenerative diseases such as cancer, arthritis, atherosclerosis, and diabetes (Cochrane, 1991; Halliwell, 1991). Aging is also accompanied by the accumulation of oxidized proteins (Stadtman and Oliver, 1991). Both membrane and intracellular biomolecules including phospholipids and proteins are readily attacked by free radicals, creating oxidized products such as MDA and carbonyls (Davies, 1987; Halliwell and Chirico, 1993, Lee and Hendricks, 1997a). In this study, both Cu (II) and AA were used to catalyze oxidative damage to deoxyribose, phospholipid, and erythrocytes. Copper can catalyze the generation of the most reactive oxygen species, hydroxyl radicals, from H_2O_2 via the Fenton reaction (Minotti and Aust, 1992).

$$Cu^{+} + H_2O_2 \longrightarrow {}^{\bullet}OH + OH^{-} + Cu^{2+}$$
$$Cu^{2+} + \text{ascorbate} \longrightarrow Cu^{+} + \text{semidehydroascorbate}$$

Carnosine, anserine, homocarnosine, and histidine effectively inhibited deoxyribose degradation and lipid peroxidation in the Cu (II) + AA-dependent system. Several mechanisms might be involved in the inhibitory effects of these compounds. First, carnosine, anserine, and homocarnosine are known to scavenge superoxide anions and peroxyl radicals (Kohen *et al.*, 1988; Boldyrev and Severin, 1990). In addition, histidine-containing dipeptides can scavenge hydroxyl radicals produced in the Fenton reaction (Chan *et al.*, 1994). In this study, the inhibitory effect of these compounds on deoxyribose degradation may be related to their hydroxyl radical scavenging activity, because hydroxyl radicals are known to break deoxyribose into MDA-like compounds (Lee and Hendricks, 1997a; 1997b). Second, carnosine and anserine are known to be very effective copper-chelating agents (Brown, 1981). When the concentration (physiologically relevant) of carnosine is 100–1000 times that of the cupric ion, four molecules of carnosine are bound to one molecule of copper (Brown, 1981). The complexes of carnosine and related compounds with copper may be very stable and catalytically inactive in liposomal lipid peroxidation. Third, carnosine and anserine act as reducing agents, thereby maintaining the reduced form of copper in the aqueous solution (Kohen *et al.*, 1988). In this study, carnosine and anserine were effective agents for the reduction of Cu (II) to Cu (I). The ratio of Cu (II) to Cu (I) was reported to be important to stimulate lipid peroxidation (Minotti and Aust, 1992). Therefore, the reduction of Cu (II) by carnosine and anserine might affect the liposomal lipid peroxidation and deoxyribose degradation. Fourth, histidine-containing dipeptides may have an enzyme-like activity. Carnosine diminished the amount of the already-formed lipid peroxidation products, so it could act as a lipid peroxidase (Babizhayev *et al.*, 1994). The complexes of copper:carnosine and copper:homocarnosine can also dismute superoxide radicals, indicating an SOD-like activity (Kohen *et al.*, 1991). Carnosine has been reported to inhibit lipid peroxidation catalyzed by iron, hemoglobin, lipoxidase, and singlet oxygen (Decker *et al.*, 1992). These results indicate that the inhibitory effects of carnosine and related compounds on lipid peroxidation or deoxyribose degradation may be due to the involvement of several mechanisms and not one single mechanism.

Carnosine, homocarnosine, anserine, and histidine also protected against metal-catalyzed HAS oxidation, as determined by carbonyl formation. Kim *et al.* (1998) reported that iron and heme-mediated Fenton-like reactions produced oxidative protein modification via formation of hydroxyl radicals and ferryl iron, respectively. In addition, lipid peroxidation products are associated with DNA damage and mutagenicity (Koh *et al.*, 1997). Therefore, the protection by histidine-containing dipetides against oxidative protein and lipid damage can be implicated in the retardation of aging or prevention of degenerative diseases such as cancer and atherosclerosis.

Ascorbic acid can be a pro-oxidant or antioxidant. The actions of AA may depend on the concentrations of AA and transition metal ions. Many *in vitro* metal/ascorbate systems have been used for free radical studies where AA acts as a pro-oxidant. A small amount of AA can increase lipid peroxidation, while high concentrations of AA inhibit the reaction (Miller and Aust, 1989; Lee and Hendricks, 1997b). Ascorbic acid, present in high concentrations in mammalian tissues, can not only scavenge active and stable oxygen radicals but can also regenerate vitamin E (Niki, 1991; Winkler *et al.*, 1994). Copper is a well-known catalyst for AA oxidation. Carnosine, anserine, homocarnosine, and histidine effectively inhibited 1 μM Cu (II)-catalyzed AA oxidation. Although the mechanism of these compounds to inhibit the AA oxidation is uncertain, it may be due to chelating copper, thereby generating less-active complexes for AA oxidation, or competing with AA for reducing Cu (II). In addition, they may inhibit AA oxidation by breaking the cascades of oxygen radical production. Carnosine and related compounds may preserve the antioxidant potential of AA, especially in the eye lens where AA prevents cataract formation (Meister, 1994).

Metal-catalyzed oxidation reactions in biological membranes impair membrane functioning, change fluidity, inactivate membrane-bound receptors and enzymes, and

increase nonspecific permeability to ions such as Ca^{2+} (Cochrane, 1991; Halliwell, 1991). The erythrocyte is a unique biological structure containing high concentrations of polyunsaturated fatty acids, cellular oxygen, and ferrous iron in the ligand state. These conditions might be expected to make it highly susceptible to oxidative damage. Copper catalyzes hemolysis of RBC by the formation of free radical and MetHb formation, depletion of glutathione levels, and interference with the reactive thiol groups of G6PDH (Hebbel, 1985; Rachmilewitz *et al.*, 1985; Kuross and Hebbel, 1988; Shinar *et al.*, 1989). Free radicals generated in the presence of Cu (II) and AA might cause hemolysis by inducing the chain oxidation reactions of lipids and proteins in the RBC membrane. Copper + ascorbic acid also enhances the oxidation of hemoglobin (Shinar *et al.*, 1989). However, MetHb formation might inhibit lipid peroxidation in the RBC membrane and eventually might prevent hemolysis because MetHb is not an effective catalyst for lipid peroxidation in the presence of H_2O_2 (Clemens *et al.*, 1985). The protection conferred by carnosine and related compounds against hemolysis may be due to either the formation of copper-complexes and reduction of Cu (II) to Cu (I) or scavenging reactive oxygen species such as superoxide anions and hydroxyl radicals. In addition, the increase in MetHb by carnosine, anserine, and histidine may be related to the protection against hemolysis, perhaps resulting from a decrease in RBC membranal lipid peroxidation.

The antioxidant activity of carnosine and related compounds may be due to the imidazole moiety of the molecules. The proton on the nitrogen of the imidazole ring is especially important for antioxidant activity. Our results suggest that histidine-containing compounds including carnosine, anserine and homocarnosine, which can be endogenously synthesized and supplied by a histidine-supplement diet, may effectively protect against free radical-mediated damage of biomolecules or cells thereby preserving their biochemical and physiological functions.

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References

Babizhayev, W. A., Seguin, M. C., Gueyne, J., Evstigneeva, R. P., Ageyeva, E. A. and Zheltukhina, G. A. (1994) L-carnosine (β-alanyl-L-histidine) and carcinine (β-alanylhistamine) act as natural antioxidants with hydroxyl-radical-scavenging and lipid-peroxidase activities. *Biochem. J.* **304**, 509–516.

Boldyrev, A. A. and Severin, S. E. (1990) The histidine-containing dipeptides, carnosine and anserine: distribution, properties and biological significance. *Adv. Enzyme Regul.* **30**, 175–194.

Boldyrev, A. A., Koldobski, A., Kurella, E., Maltseva, V. and Stvolinski, S. (1993) Natural histidine-containing dipetide carnosine as a potent hydrophilic antioxidant with membrane stabilizing function. A biomedical aspect. *Mol. Chem. Neuropathol.* **19**, 185–192.

Brown, C. E. (1981) Interactions among carnosine, anserine, ophidine and copper in biochemical adaptation. *J. Theor. Biol.* **88**, 245–256.

Brownlee, N. R., Huttner, J. J., Panganamala, R. V. and Cornwell, D. G. (1977) Role of vitamin E in glutathione-induced oxidant stress: metmyoglobin, lipid peroxidation, and hemolysis. *J. Lipid Res.* **18**, 635–644.

Chan, W. K. M., Decker, E. A., Lee, J. B. and Butterfield, D. A. (1994) EPR spin-trapping studies of the hydroxyl radical scavenging activity of carnosine and related dipeptides. *J. Agric. Food Chem.* **42**, 1407–1410.

Chapman, M. L., Rubin, B. R. and Gracy, R. W. (1989) Increased carbonyl content of proteins in synovial fluid from patients with rheumatoid arthritis. *J. Rheumatol.* **16**, 15–20.

Clemens, M. R., Einsele, H., Remmer, H. and Waller, H. D. (1985) An essential requirement for ferrous-hemoglobin in the hydrogen peroxide stimulated oxidation of red blood cell membrane lipids. *Biochem. Pharmacol.* **34**, 1339–1341.

Cochrane, C. G. (1991) Cellular injury by oxidants. *Am. J. Med.* **91**, S23–S30.

Crush, K. G. (1970) Carnosine and related substances in animal tissues. *Comp. Biochem. Physiol.* **34**, 3–30.

Davies, K. J. A. (1987) Protein damage and degradation by oxygen radicals. I. general aspect. *J. Biol. Chem.* **262**, 9895–9901.

Decker, E. A., Crum, A. D. and Calvert, J. T. (1992) Differences in the antioxidant mechanism of carnosine in the presence of copper and iron. *J. Agric. Food Chem.* **40**, 756–759.

Flancbaum, L., Fitzpatrick, J. C., Brotman, D. N., Marcoux, A. M., Kasziba, E. and Fisher, H. (1990) The presence and significance of carnosine in histamine-containing tissues of several mammalian species. *Agents Actions* **31**, 190–196.

Halliwell, B. (1991) Reactive oxygen species in living systems: source, biochemistry, and role in human disease. *Am. J. Med.* **91**, S14–S22.

Halliwell, B. and Chirico, S. (1993) Lipid peroxidation: its mechanism, measurement and significant. *Am. J. Clin. Nutr.* **57**, 715–725.

Harris, R. C., Marlin, D. J., Dunnet, M., Snow, D. H. and Hultman, E. (1990) Muscle buffering capacity and dipeptide content in the thoroughbred horse, greyhound dog and man. *Comp. Biochem. Physiol.* **97A**, 249–251.

Hebbel, R. P. (1985) Auto-oxidation and a membrane-associated "Fenton reagent"; A possible explanation for development of membrane lesions in sickle erythrocytes. *Clin. Hematol.* **14**, 129–140.

Hipkiss, A. R., Preston, J. E., Himsworth, D. T., Worthington, V. C., Keown, M., Michaelis, J., Lawrence, J., Mateen, A., Allende, L., Eagle, P. A. and Abbott, N. J. (1998) Pluripotent protective effects of carnosine, a naturally occurring dipeptides. *Ann. NY Acad. Sci.* **854**, 37–53.

Ikeda, J., Kimura, T. and Tamaki, N. (1980) Activation of rabbit muscle fructose 1,6-bisphosphatase by histidine and carnosine. *J. Biochem.* **87**, 179–185.

Jackson, M. C. and Lenney, J. F. (1996) The distribution of

carnosine and related dipeptides in rat and human tissues. *Inflamm. Res.* **45**, 132–135.

Kim, Y. M., Kim, S. S., Kang, G., Yoo, Y. M., Kim, K. M., Lee, M. E., Han, J. A. and Hong, S. J. (1998) Comparative studies of protein modification mediated by fenton-like reactions of iron, hematin, and hemoglobin: generation of different reacive oxidizing species. *J. Biochem. Mol. Biol.* (formerly *Korean Biochem. J.*) **31**, 161–169.

Koh, Y. H., Yoon, S. J. and Park, J. W. (1997) Lipid peroxidation product-mediated DNA damage and mutagenicity. *J. Biochem. Mol. Biol.* (formerly *Korean Biochem. J.*) **30**, 188–193.

Kohen, R., Misgav, R. and Ginsburg, I. (1991) The SOD like activity of copper:carnosine, copper:anserine and copper:homocarnosine complexes. *Free Radic. Res. Commun.* **12-13**, 179–185.

Kohen, R., Yamamoto, Y., Cundy, K. C. and Ames, B. N. (1988) Antioxidant activity of carnosine, homocarnosine, and anserine present in muscle and brain. *Proc. Natl. Acad. Sci. USA* **85**, 3175–3179.

Kuross, S. A. and Hebbel, R. P (1988) Nonheme iron in sickle erythrocyte membrane: Association with phospholipids and potential role in lipid peroxidation *Blood* **72**, 1278–1285.

Lee, B. J., Hendricks, D. G. and Cornforth, D. P. (1998a) A comparison of carnosine and ascorbic acid on color and lipid stability of ground beef. *Meat Sci.* **51**, 245–253.

Lee, B. J., Hendricks, D. G. and Cornforth, D. P. (1998b) Antioxidant effects of carnosine and phytic acid in a model beef system. *J. Food Sci.* **63**, 394–398.

Lee, B. J. and Hendricks, D. G. (1997a) Antioxidant effects of L-carnosine on liposomes and beef homogenates. *J. Food Sci.* **62**, 931–934.

Lee, B. J. and Hendricks, D. G. (1997b) Metal-catalyzed oxidation of ascorbate, deoxyribose and linoleic acids as affected by phytic acid in a model system. *J. Food Sci.* **62**, 935–938.

Meister, A. (1994) Glutathione-ascorbic acid antioxidant system in animals. *J. Biol. Chem.* **269**, 9397–9400.

Miller, D. M and Aust, S. D. (1989) Studies of ascorbate-dependent, iron-catalyzed lipid peroxidation. *Arch. Biochem. Biophys.* **271**, 113–119.

Minotti, G. and Aust, S. D. (1992) Redox cycling of iron and lipid peroxidation. *Lipids* **27**, 219–226.

Niki, E. (1991) Action of ascorbic acid as a scavenger of active and stable oxygen radicals. *Am J. Clin. Nutr.* **54**, 1119s–1124s.

Park, D. K. and Song, J. H. (1994) Determination of the antioxidant activity of vitamins C and E according to lipid physical state using high performance liquid chromatography with chemiluminescence detection. *Korean Biochem. J.* (presently *J. Biochem. Mol. Biol.*) **27**, 473–478.

Parker, C. J. and Ring, E. (1970) A comparative study of the effect of carnosine on myofibrillas-ATPase activity of vertebrate and invertebrate muscle. *Comp. Biochem. Physiol.* **37**, 413–419.

Rachmilewitz, E. A., Shinar, E., Shalev, O., Galili, U. and Schrier, S. L. (1985) Erythrocyte membrane alterations in beta-thalassemia. *Clin. Hematol.* **14**, 163–182.

Reznick, A. Z. and Packer, L. (1994) Oxidative damage to protein: Spectrophotometric method for carbonyl assay. *Methods Enzymol.* **233**, 357–363.

Reznick, A. Z., Kagan, V. E., Ramsey, R., Tsuchiya, M., Khawaja, S., Servinova, E. A. and Packer, L. (1992) Antiradical effects in L-propionyl carnitine protection of the heart against ischemia-reperfusion injury: The possible role of iron chelation. *Arch. Biochem. Biophys.* **296**, 394–401.

Shinar, E., Rachmilewitz, E. A., Shifter, A., Rahamim, E. and Saltman, P. (1989) Oxidative damage to human red cells induced by copper and iron complexes in the presence of ascorbate. *Biochim. Biophys. Acta* **1014**, 66–72.

Stadtman, E. R. and Oliver, C. N. (1991) Metal-catalyzed oxidation of proteins. Physiological consequences. *J. Biol. Chem.* **266**, 2005–2008.

Williams, H. L., Johnson, D. J. and Haut, M. J. (1977) Simultaneous spectrophotometry of Fe^{2+} and Cu^{2+} in serum denatured with guanidine hydrochloride. *Clin. Chem.* **23**, 237–240.

Winkler, B. S., Orselli, S. M. and Rex, T. X. (1994) The redox couple between glutathione and ascorbic acid: A chemical and physiological perspective. *Free Radic. Biol. Med.* **17**, 333–349.

Wolff, S. P. (1994) Ferrous ion oxidation in the presence of ferric ion indicator xylenol orange for measurement of hydroperoxides. *Methods Enzymol.* **233**, 182–189.